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# Investigation of the Adsorption of Dioleoyl-L- $\alpha$ -phosphatidic Acid Mono- and Bilayers from Vesicle Solution onto Polyethylenimine-Covered Substrates

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The formation of monolayers and bilayers of dioleoyl-L- $\alpha$ -phosphatidic acid from solutions of small unilamellar vesicles onto polyethylenimine-treated substrates was investigated by means of small angle X-ray scattering and X-ray photoelectron spectroscopy. The formation of monolayers could be verified after an adsorption time of 5 min, on removal of the substrates from the vesicle solution and washing with water. No adsorption of dioleoyl-L- $\alpha$ -phosphatidic acid takes place when the substrates used have not been pretreated with polyethylenimine. This suggests that the interaction of the charges of polyethylenimine with the phosphatidic acid headgroups is the driving force for the adsorption. Further experiments show that immersion of the supported bilayers in polyethylenimine solution again leads to dried films with layer thicknesses of approximately twice the values of the supported monolayers. From this observation the formation of supported bilayers of lipid/polyelectrolyte complexes is derived.

## Introduction

Supported lipid bilayers have attracted attention as biomembrane models, for example for the reconstitution and investigation of peripheral and integral membrane proteins and for the study of adsorption processes on model membranes.<sup>1–3</sup> In order to be comparable with natural membranes, supported bilayers are required in a fluid phase. Such systems cannot easily be created by means of the conventional Langmuir–Blodgett technique. A good transfer onto solid supports, at least for fatty acids and phospholipids, can in most cases only be achieved when the monolayers at the air/water interface are in a solid phase. Additional problems with the Langmuir–Blodgett technique arise, when integral membrane proteins have to be incorporated in monolayers at the air/water interface and in mono- and multilayers on solid supports. As these proteins are isolated and solubilized in an aqueous detergent solution, they cannot be spread onto the air/water interface in the same manner as phospholipids and fatty acids (in a solvent immiscible with water). Therefore drops of the aqueous solution are injected beneath a preformed phospholipid monolayer or spread on a preformed phospholipid monolayer.<sup>4,5</sup> In both cases, detergent is incorporated into the monolayer at the air/water interface and after transfer to solid supports into the Langmuir–Blodgett films and influences the properties of both types of ultrathin films. The fusion of vesicles onto solid substrates from solution has been established as an alternative method for the formation of supported bilayers. This method can be used to create the whole

supported bilayer<sup>1,3,6–11</sup> or to form the second monolayer on a preformed monolayer. The latter can be created by means of the Langmuir–Blodgett technique,<sup>8,12</sup> by self-assembly methods as in the adsorption of thiols on gold,<sup>13–16</sup> or by covalent fixation on solid particles.<sup>2</sup> The method of creating supported bilayers by means of spreading liposomes should also be suitable for the immobilization of integral membrane proteins into lipid bilayers on solid supports. First the membrane protein is reconstituted by a standard dialysis technique into vesicles before being spread onto the solid substrate from solution.<sup>2</sup> This method has the advantage that the detergent is removed through the dialysis. For a better control of such immobilization techniques, the process by which the liposomes are spread onto the surface of solid substrates has to be investigated in detail.

It has already been reported that certain polyelectrolytes induce fusion of vesicles.<sup>17–19</sup> Complexes of polyelectrolyte and phospholipid bilayers with a lamellar structure can be produced for certain components and conditions.<sup>20,21</sup> Such lamellar structures of charged lipids between

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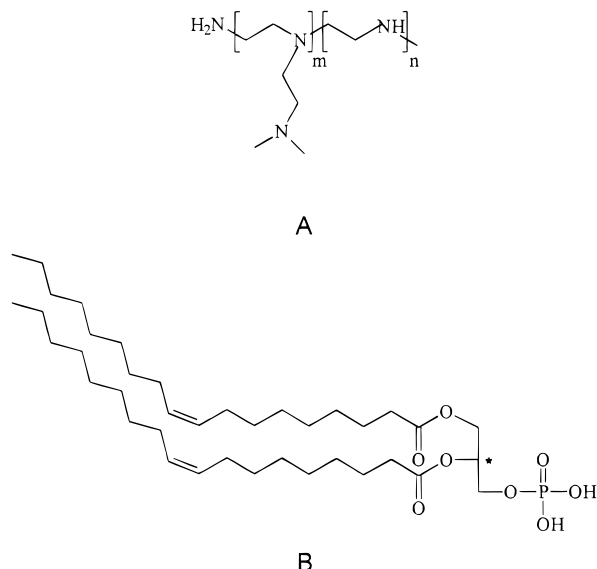
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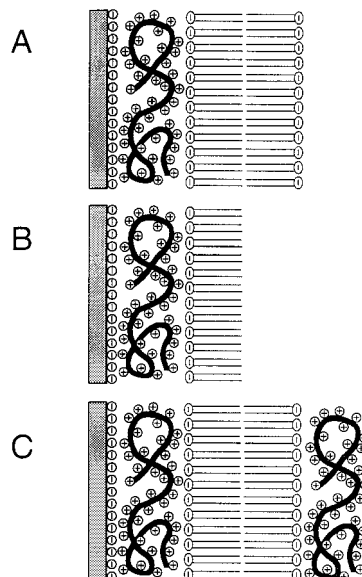


**Figure 1.** Chemical structures of (A) polyethylenimine and (B) dioleoyl-L- $\alpha$ -phosphatidic acid.

polyelectrolyte layers have also been prepared by means of the Langmuir–Blodgett technique.<sup>22,23</sup> In this work the possibility of forming such lamellar structures at a surface of the solid substrate by successive adsorption of the components from polyelectrolyte and vesicle solutions was investigated. Polyethylenimine (PEI, Figure 1A), which behaves as polyelectrolyte due to protonation of the amino groups in aqueous solutions, was chosen, as it has been demonstrated that this forms defined adsorption layers on glass substrates.<sup>24</sup>

In addition, the interaction of this material with charged amphiphiles has already been extensively investigated in monolayers at the air/water interface, in Langmuir–Blodgett monolayers,<sup>23–26</sup> and in monolayers on solid substrates formed by means of adsorption from detergent solution.<sup>27</sup> Dioleoyl-L- $\alpha$ -phosphatidic acid (DOPA, Figure 1B) was chosen, as it is negatively charged and exhibits a fluid phase already at room temperature.<sup>28</sup> It was expected that the interaction of the opposite charges of the polyethylenimine and dioleoyl-L- $\alpha$ -phosphatidic acid in solutions of liposomes would lead to the formation of supported bilayers at the substrate. This is schematically depicted in Figure 2A.

Such supported bilayers of phospholipids with the headgroups on top are supposed to be stable only whilst being immersed in an aqueous phase. When they are removed from the aqueous phase, the top layer is floated off and the supported monolayer is left (Figure 2B). First the structure of these supported monolayers is investigated as an indication that fusion of the vesicles with the surface has occurred. In a further series of experiments it is investigated whether a further polyethylenimine layer can be adsorbed on top of the supported bilayers to form the structure which is depicted in Figure 2C and how such a system behaves on removal from the aqueous phase. The structures of supported monolayers and bilayers have



**Figure 2.** Schematic representations of the supramolecular structures on solid substrates, discussed in this article. The polyelectrolyte represents polyethylenimine; the phospholipid represents dioleoyl-L- $\alpha$ -phosphatidic acid. Counterions (hydroxide ions) have been omitted for clarity: (A) supported monolayer on a single polyelectrolyte layer; (B) supported monolayer between single polyelectrolyte layers; (C) supported monolayer between polyelectrolyte layers.

been characterized by means of X-ray reflectivity measurements. X-ray photoelectron spectroscopy measurements were performed in addition.

## Experimental Section

**Materials.** Dioleoyl-L- $\alpha$ -phosphatidic acid sodium salt (synthetic, purity approximately 98%) and polyethylenimine (average molecular weight: 50 000) as 50% w/w aqueous solutions were purchased from Sigma. According to the information provided by the manufacturer, the polyethylenimine is branched and contains primary, secondary, and tertiary amino groups in the ratio 1:2:1. All chemicals were used as received without further purification. In all experiments including washing steps, the water was purified by reversed osmosis (Elgastat reversed osmosis system), followed by ion-exchange and filtration steps with a Milli-Q purification system. The resistivity was better than 18.2 M $\Omega$ -cm, and the total organic content was less than 10 ppb (according to the manufacturer). The pH value of the water was 5.0.

**Substrates and Their Purification.** Polished silicon wafers were employed as substrates. These were purchased from Topsil Semiconductor Materials A/S, Frederikssund, Denmark. The purification of the wafers was carried out with a modified RCA cleaning procedure.<sup>29</sup> This started with an alkaline cleaning step, in which the samples were heated in a  $\text{H}_2\text{O}/\text{H}_2\text{O}_2/\text{NH}_3$  (5:1:1) mixture (v/v/v) to 75–80 °C for 10 min. After rinsing with Milli-Q water for 5 min, the substrates were sonicated for 10 min in a 6:1 mixture of concentrated HCl and Milli-Q water in a sonicator bath and extensively rinsed with water again.

**Vesicle Preparation.** Dioleoyl-L- $\alpha$ -phosphatidic acid sodium salt was suspended in Milli-Q water in a concentration of 5 mg/mL, followed by 10 min of shaking with a flask shaker. This suspension was sonicated for 20 min under nitrogen atmosphere with a Branson Model 250 tip sonicator equipped with a 1/8 in. microtip, operating in the pulsed mode with 50% duty cycle at 55 W output. After this procedure a clear solution was obtained. For the adsorption experiments the vesicle solution was diluted to a final concentration of 0.2 mg/mL with Milli-Q water.

**Adsorption Experiments.** The adsorption of a polyethylenimine layer on the solid substrates was performed by immersion of the hydrophilic, cleaned substrates in a solution of polyethylenimine with a concentration of  $3 \times 10^{-2}$  mol/L for 30 min,

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exhibiting a pH value of 10.5. After each adsorption step the samples were immersed three times in 30 mL of water for 1 min. After the treatment with the polyelectrolytes, the samples still were completely hydrophilic. The samples were transferred into the vesicle solution for the adsorption of dioleoyl-L- $\alpha$ -phosphatidic acid. To prepare supported monolayers, samples were removed from the vesicle solution at different times (5, 20, and 40 min) and then washed three times with 20 mL of water for 1 min. At this point, it could already be observed that the samples had become hydrophobic, as only a few drops of water remained on the surface on removal from the water, indicating a good coverage of the surface with the phospholipid monolayer. To prepare stabilized supported bilayers (see Figure 1C), the majority of the vesicle solution was removed with a pipet in such a way that the samples remained below the surface of the solution. Then 80 mL of water was added and 80 mL of the solution was removed after a minute. This procedure was repeated at least three times. Then 40 mL of the polyethylenimine solution was added. After 20 min the samples were washed three times in Milli-Q water and blown dry with nitrogen gas. Most of the samples exhibited a nonuniform wetting behavior, after removal from the polyethylenimine solution. Hydrophobic as well hydrophobic patches were visible at the layer surfaces, suggesting a nonuniform surface.

**Preparation of Bulk Samples.** To determine the layer spacing of dioleoyl-L- $\alpha$ -phosphatidic acid sodium salt in the bulk phase, a few milligrams of the compound were smeared onto a glass plate. To investigate the structures which were formed in solution, 0.5 mL of the liposome solution used for the preparation of the adsorption films was applied on a glass slide and air dried.

**Small Angle X-ray Reflectivity.** All measurements were performed on a Siemens D 500 diffractometer in the  $\theta/2\theta$  geometry equipped with a graphite monochromator at the detector side, employing copper K $\alpha$  radiation of 1.542 Å and employing two slits with opening angles of 0.1° between the tube and the samples and slits of 0.1° and 0.018° between the samples and the monochromator. The tube was operated with 35 mA and 40 kV. The scattered intensity of the background was measured with the sample ( $\theta$ -circle) rotated 0.3° out of the specular condition and was subtracted from the specular scattered intensity after being smoothed over five measuring points. The analysis of the X-ray measurements was performed, using the kinematic approximation. The reflectivity,  $R$ , divided by the Fresnel reflectivity,  $R_F$ , for an ideal interface is given by<sup>30–32</sup>:

$$\frac{R}{R_F} = \frac{1}{\rho_s^2} \left| \int \frac{d\rho}{dz} e^{iQ_z z} dz \right|^2 \quad (1)$$

$\rho_s$  is the electron density of the substrate,  $d\rho/dz$  is the derivative of the electron density profile, and  $Q_z = (4\pi/\lambda) \sin(\theta)$  is the scattering vector. To model the electron density distribution along the  $z$ -direction, the film was divided into slabs (boxes) with thicknesses  $d_i$  and electron densities  $\rho_i$ . The interface roughnesses were introduced by Gaussian smearings,  $\sigma_i$ , of the densities at the interface between slabs  $i$  and  $i+1$ . Then the integral in eq 1 transforms into<sup>31,32</sup>

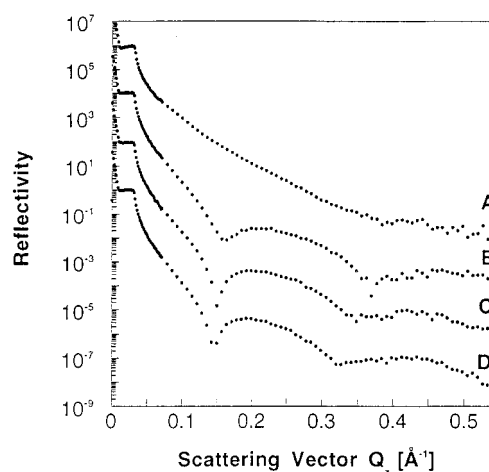
$$\frac{R}{R_F} = \frac{1}{\rho_s^2} \left| \sum_{i=0}^N \left( \rho_i - \rho_{i+1} \right) e^{-iQ_z d_i} e^{-Q_z^2 \sigma_{i+1}^2 / 2} \right|^2 \quad (2)$$

**X-ray Photoelectron Spectroscopy (XPS).** X-ray photoelectron spectra were measured with a commercially available "top hat" X-Probe 300 from Surface Science Instruments, using monochromated aluminum K $\alpha$ -radiation with an energy of 1486.6 eV. The background pressure was in the low  $10^{-10}$  mbar range. The used experimental resolution was 1.8 eV.

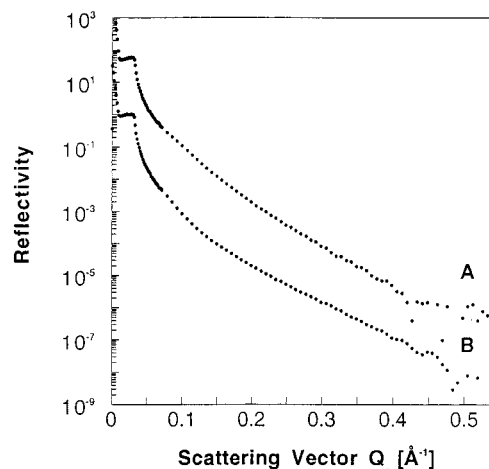
## Results and Discussion

### 1. Supported Monolayers on Polyethylenimine-Treated Substrates. X-Ray Reflectivity.

Figure 3A



**Figure 3.** X-ray reflectivity curves of adsorption films: (A) silicon wafer exposed to a solution of polyethylenimine for 30 min; (B–D) silicon wafers exposed to a solution of polyethylenimine for 30 min and after that to a solution of dioleoyl-L- $\alpha$ -phosphatidic acid vesicles for 5 min (B), 20 min (C), and 40 min (D). Successive data sets are displaced by multiplication by 100.



**Figure 4.** X-ray reflectivity curves of two hydrophilic silicon wafers exposed to a solution of dioleoyl-L- $\alpha$ -phosphatidic acid vesicles for 20 min (A) and 40 min (B). Curve A is displaced by multiplication by 100.

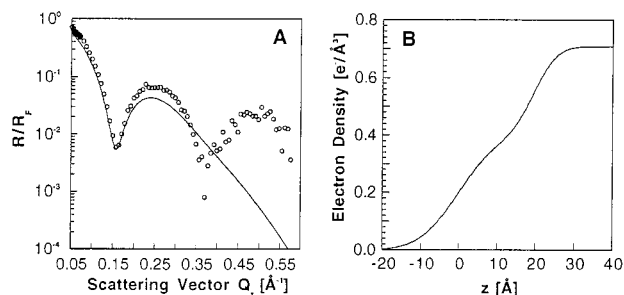
shows the X-ray reflectivity of silicon wafers exposed to a solution of polyethylenimine. No modulations are detectable, suggesting a very thin film. Figure 3B–D depicts scattering curves of silicon wafers treated with polyethylenimine exposed to the vesicle solution for 5, 20, and 40 min. For comparison, the scattering curves of untreated silicon samples are shown in Figure 4 being exposed for 20 (A) and 40 min (B) to the vesicle solution. As can be seen from Figures 3 and 4, only the scattering curves of those silicon wafers which were treated with polyethylenimine exhibit oscillations due to an adsorbed layer. Therefore, it can already be concluded that adsorption from vesicle solution only took place on polyelectrolyte-treated surfaces.

For a first discussion of the structure of the adsorption layer, it can be assumed that it consists of a slab of thickness  $D$  with constant electron density  $\rho$ . In this case the total film thickness is given by  $\pi/Q_{\min}$  with  $Q_{\min}$  being the position of the first minimum of the scattering curve.<sup>30,31</sup> For the three samples, approximate values of the total thickness are 19, 21, and 20 Å. These values correspond to a monolayer of phospholipid on a thin layer of polyelectrolyte.

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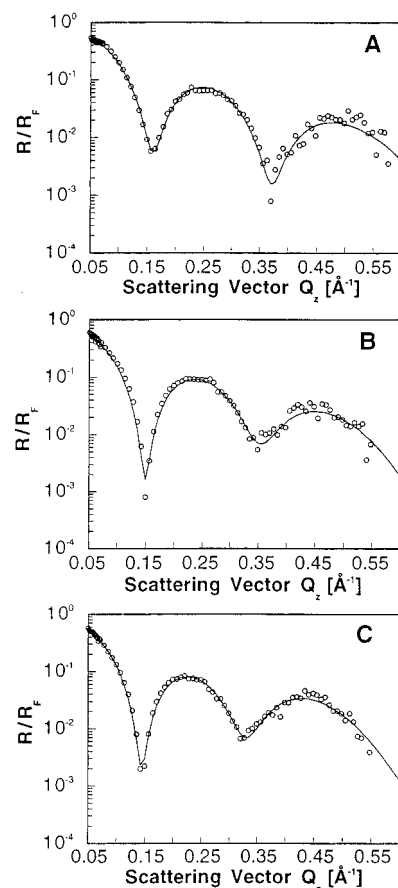


**Figure 5.** Best one-slab fit to the X-ray reflectivity curve of the sample being exposed to a solution of polyethylenimine for 30 min and after that to a solution of dioleoyl-L- $\alpha$ -phosphatidic acid vesicles for 5 min (curve B in Figure 3).

For the simulation of the diffractograms, the surface roughness of the silicon interface was determined from separate reflectivity measurements on three bare silicon wafers which were exposed to the same cleaning conditions. An average value of  $4.6 \pm 0.7$  Å was obtained. This value together with the value for the electron density of silicon was fixed for all curve fits. A separate SiO<sub>2</sub> layer was not taken into account to simulate the diffractograms, as measurements of the bare silicon wafers cleaned in the same way as the ones used for adsorption experiments in the same scattering vector range showed that the thickness of such a layer could not be resolved (upper curve in Figure 3). Also the results of the curve fits of the scattering curves of the supported monolayers showed that good fits could be obtained without taking an oxide layer into account. At first glance this seems to be surprising, as e.g. in the investigation of ultrathin chemisorption films by means of X-ray reflectivity a natural oxide layer with a thickness of 17 Å on silicon could be detected.<sup>31,32</sup> The following explanations are given for the result that the oxide layer could not be resolved by means of X-ray reflectivity measurements in this work: The electron densities of silicon and silicon dioxide are very similar ( $\rho_{\text{oxide}}/\rho_{\text{silicon}} = 0.95$ ).<sup>31,32</sup> Therefore this layer gives only a small contribution to the reflectivity of the wafer, and the minimum of the reflectivity curve of bare silicon wafers resulting from the thin oxide layer is very shallow and appears at high scattering vectors. This is difficult to detect with the setup used for this study, which is a clear modified commercially available spectrometer. In addition our cleaning procedure seems to etch away substantial parts of the oxide layer. This is supported by the results of another study concerning SAXR on adsorbed polyelectrolyte films on silicon.<sup>33</sup> There the same cleaning procedure was employed and the SiO<sub>2</sub> layer could not be resolved too, although the measurements were performed using a rotating anode X-ray generator.

Simulations were at first carried out with one-slab models. The best one-slab fit of the scattering curve B in Figure 3 is depicted as an example in Figure 5. This fit gives a total film thickness of  $20 \pm 2$  Å, an electron density of  $0.40 \pm 0.13$  e<sup>-</sup>/Å<sup>3</sup> for the film, and a roughness for the film/air interface of  $8 \pm 2$  Å. Figure 5 shows that a one-slab fit is insufficient at high scattering vectors.

In the following, the results of two-slab fits on the reflectivity curves are presented and discussed. The first slab is assumed for the polyethylenimine layer and the phospholipid headgroups, the second slab for the alkyl chains of the phospholipid. It could be argued that a three-slab model is necessary, one for the polyethylenimine layer, one for the phospholipid headgroups, and one for the alkyl chains, the headgroup slab exhibiting the highest electron

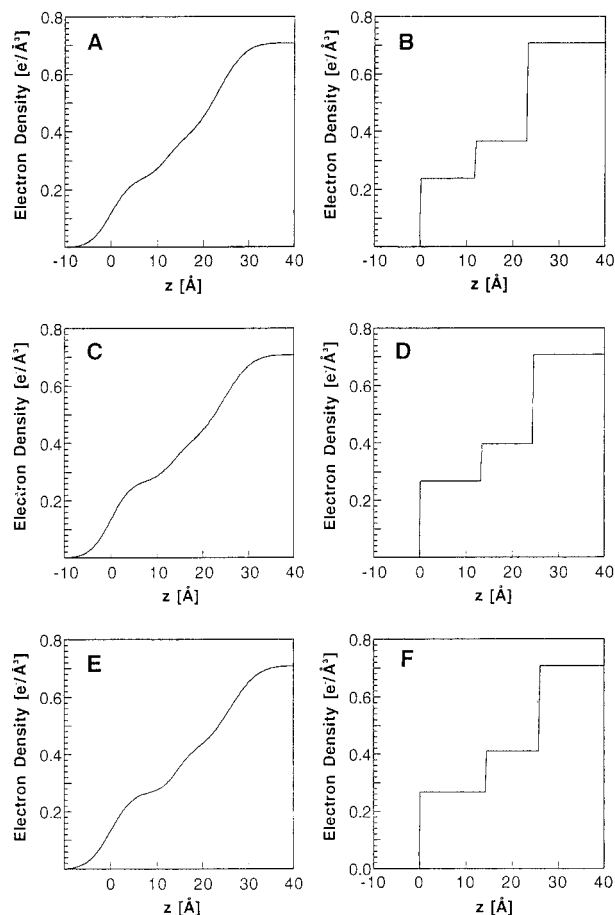


**Figure 6.** Two-slab fits and normalized reflectivity curves from Figure 3B–E: Silicon wafers exposed to a solution of polyethylenimine for 30 min and after that to a solution of dioleoyl-L-phosphatidic acid vesicles for 5 min (A), 20 min (B), and 40 min (C).

density. But from Figure 3A it can already be derived that the polyelectrolyte layer at the surface of the silicon substrate should be very thin, as the diffractogram does not exhibit any oscillations. This may be the reason why two different slabs for polyelectrolyte and headgroups could not be distinguished. The best fits for all three samples are shown in Figure 6. The corresponding electron density profiles are depicted in Figure 7A, C, and E. To visualize the average electron densities and the thicknesses of the slabs, the same electron density profiles are depicted in Figure 7B, D, and F, assuming that all interface roughnesses are zero. The fit parameters are listed in Table 1.

As Table 1 shows, this model gives a slightly greater film thickness than can be derived from a one-slab model. Considering the first slab, consisting of PEI and phosphatidic acid headgroups, no systematic relation between layer thickness or electron density and adsorption time can be observed. A value for the electron density is expected which is a weighted average of the values for the phosphatidic acid headgroup and polyethylenimine. Up to now, no results from X-ray reflectivity measurements on polyethylenimine layers have been reported in the literature. For a multilayer of polyallylamine and polystyrene sulfonate a value of  $0.403$  e<sup>-</sup>/Å<sup>3</sup> has been reported.<sup>33</sup> There the adsorption experiments were carried out with solutions containing 2 M NaCl. It follows that a portion of these ions may have been incorporated into the adsorption layers, thereby increasing their electron density. A lower value for polyethylenimine is to be expected, because it contains less heavy atoms and contains less unsaturated groups, as here the adsorption

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**Figure 7.** Corresponding electron density profiles to the curve fits in Figure 5. A, C, and D depict the electron density profiles corresponding to the curve fits in Figure 5A–C. For clarity the same electron density profiles are shown in parts B, D, and E assuming all interface roughnesses to be zero.

of the polyelectrolyte was carried out using salt free solutions. For this reason the above-mentioned value can be taken as an upper limit for the electron density of the polyethylenimine layer. As a lower limit, a value of  $0.334 \text{ e}^-/\text{\AA}^3$  can be taken, as this is the value for water and for the ethylamine part of phospholipids<sup>34</sup> and polyethylenimine consists of such units. X-ray reflectivity measurements have not been reported for a phospholipid with phosphatidic acid headgroups either. Nevertheless electron density values for the headgroups of phosphatidylcholines or phosphatidylethanolamines can be used for comparison, as only the ester groups and the phosphate groups contribute to the slab for the headgroup, whereas the ethylamine groups exhibit almost the same electron density as water.<sup>34</sup> In monolayers of L- $\alpha$ -DMPE (L- $\alpha$ -dimyristoylphosphatidylethanolamine) at the air/water interface electron densities of 0.401 and  $0.515 \text{ e}^-/\text{\AA}^3$  in the liquid-expanded and solid state, respectively, have been reported.<sup>34</sup> For monolayers of L- $\alpha$ -DLPE (L- $\alpha$ -dilauroylphosphatidylethanolamine) the corresponding values are 0.414 and  $0.525 \text{ e}^-/\text{\AA}^3$  in the liquid-expanded state and solid state, respectively.<sup>34</sup> According to the above arguments, the electron density for the first slab of the supported monolayers should exhibit values between  $0.334$  and  $0.515 \text{ e}^-/\text{\AA}^3$ . Therefore, the determined values in Table 1 are reasonable.

The question arises whether the thickness of the headgroups and the polyethylenimine layer can be derived.

Little data may be found in the literature about X-ray reflectivity studies on phosphatidic acids. Therefore the values for the thickness of the first slab are compared with the thickness of the headgroups of other phospholipids in monolayers at the air/water interface of L- $\alpha$ -DMPE and L- $\alpha$ -DLPE and crystals of *rac*-DLPE. As already mentioned, in the case of reflectivity measurements on L- $\alpha$ -DMPE monolayers, only the glycerol ester groups and phosphate groups contribute to the headgroup slab. Therefore, these values can be compared directly with a phosphatidic acid. For an L- $\alpha$ -DMPE monolayer at the air/water interface, values between 11.07 (liquid-expanded phase) and 7.55 Å (solid phase) have been reported, and for an L- $\alpha$ -DLPE monolayer, these values are between 11.80 Å (liquid-expanded phase) and 7.72 Å (solid phase).<sup>33</sup> In a crystal of *rac*-DLPE the headgroup length is 5.5 Å.<sup>35,36</sup> A thickness of the headgroups of 11 Å would leave no space for the polyethylenimine layer. As the presence of the latter can be shown by means of ESCA measurements (see below), one can conclude that the arrangement and conformation of the headgroups might be similar to the one in the solid state of L- $\alpha$ -DMPE and L- $\alpha$ -DLPE in monolayers at the air/water interface or in crystals of *rac*-DLPE. From the difference of the thickness  $d_1$  of the first slab (PEI + headgroups) and a minimum thickness of the headgroup layer (5.5 Å) a maximum thickness for the polyethylenimine layer between 5.7 Å ( $t_{\text{ads}} = 20 \text{ min}$ ) and 6.1 Å ( $t_{\text{ads}} = 40 \text{ min}$ ) can be estimated. This is similar to the values reported for other polyelectrolyte layers. For adsorption layers of polyvinylpyridine on mica, thicknesses of 6 Å have been derived from surface force measurements.<sup>37</sup> For a layer pair consisting of an adsorption layer of poly(vinyl sulfate) and polyallylamine a thickness of 6.45 Å per layer (13 Å per layer pair) has been measured.<sup>38</sup> The low thickness of the adsorption layer of polyethylenimine and the other mentioned polyelectrolytes can be explained by the fact that the solutions contained no additional ions. This leads to an adsorption of the polyelectrolytes in a rather flat conformation onto the solid surfaces, as the charged groups of the polyelectrolyte repel each other and tend to maximize the distance from each other. In the presence of additional counterions, the charges of the polyelectrolyte are screened and the polyelectrolyte exhibits a more coiled conformation, leading to a thicker adsorption layer. This has been analyzed theoretically by van der Schree and Lyklema.<sup>39</sup> The same type of behavior has been found for multilayers of oppositely charged polyelectrolytes in the experiments of Decher and co-workers.<sup>38,40</sup>

As Table 1 shows, the thickness of the alkyl chain layer increases slightly with time from 11.8 to 14.1 Å. Together with the increase of the electron density of the slab for the headgroup and polyethylenimine a slight increase of the packing density of the phospholipid layer can be derived with time. The electron density for the alkyl chains at an adsorption time of 5 min is lower than that at 20 and 40 min. This might be explained by a larger amount of defects. The electron densities of the samples with adsorption times of 20 and 40 min are equal and are typical for systems with fluid alkyl chains: for hexadecane  $0.267 \text{ e}^-/\text{\AA}^3$  in the bulk phase can be calculated from the

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**Table 1. Fit Parameters for the Best Representations of the Reflectivity Curves in Figure 6<sup>a</sup>**

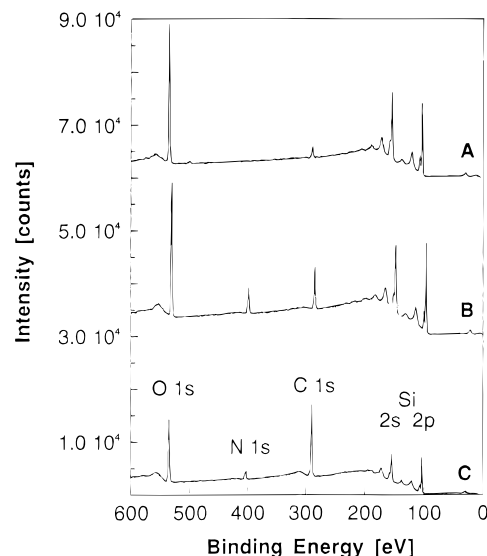
	sample 1 ( $t_{\text{ads}} = 5$ min)	sample 2 ( $t_{\text{ads}} = 20$ min)	sample 3 ( $t_{\text{ads}} = 40$ min)
layer thickness ( $\text{\AA}$ )			
L <sub>1</sub> (PEI + headgroups)	$11.3 \pm 0.4$	$11.2 \pm 0.4$	$11.6 \pm 0.3$
L <sub>2</sub> (alkyl chains)	$11.8 \pm 0.3$	$13.3 \pm 0.4$	$14.3 \pm 0.3$
total thickness	$23.1 \pm 0.7$	$24.5 \pm 0.8$	$25.9 \pm 0.6$
electron density $\rho_i$ ( $\text{e}^-/\text{\AA}^3$ )			
$\rho_0$ (silicon)	$0.705^b$	$0.705^b$	$0.705^b$
$\rho_1$ (PEI + headgroups)	$0.366 \pm 0.010$	$0.397 \pm 0.013$	$0.410 \pm 0.008$
$\rho_2$ (alkyl chains)	$0.238 \pm 0.008$	$0.268 \pm 0.010$	$0.268 \pm 0.007$
interface roughness ( $\text{\AA}$ )			
$\sigma_{01}$ (silicon/PEI)	$4.7 \pm 0.6^b$	$4.7 \pm 0.6^b$	$4.7 \pm 0.6^b$
$\sigma_{12}$ (headgroups/chains)	$3.0 \pm 0.3$	$3.1 \pm 0.5$	$2.8 \pm 0.3$
$\sigma_{23}$ (alkyl chains/air)	$3.4 \pm 0.2$	$3.5 \pm 0.2$	$3.8 \pm 0.2$

<sup>a</sup> The error of a certain parameter gives the value by which the parameter can be varied in order to increase the  $\chi^2$  value of the fit by 30%, provided that the remaining parameters are fitted again to balance the parameter under test. <sup>b</sup> Values kept fixed for the curve fits.

density.<sup>41</sup> For monolayers of DMPE at the air/water interface in the liquid-expanded state values between 0.288 and  $0.291 \text{ e}^-/\text{\AA}^3$  have been reported.<sup>34</sup>

Finally, the thicknesses of the adsorption layers will be compared with the layer periodicity of multilayer systems of DOPA. No data of the elementary cell of dioleoylphosphatidic acid or the sodium salt could be obtained from the literature. In order to compare the structures of the ultrathin films with bulk structures, X-ray diffraction measurements were performed on dioleoylphosphatidic acid sodium salt smeared onto a glass plate and on samples of the dried vesicle suspension. In both cases four orders of (00 $l$ ) reflections could be detected and the bilayer spacings could be determined as 48.1 and 49.9  $\text{\AA}$  for the dried vesicle suspension and the bulk phase of dioleoyl-L- $\alpha$ -phosphatidic acid sodium salt, respectively. Therefore, the thickness of one monolayer of the phospholipid can be calculated as 24.1  $\text{\AA}$  for dried vesicles and as 24.5  $\text{\AA}$  for the bulk sodium salt, being almost equal to the total thickness of the adsorption film. This suggests that, at least in the supported monolayers created by means of adsorption from liposome solutions on polyethylenimine, the tilt angle of the alkyl chains of the phospholipid molecules must be greater than that in the bilayer structure.

**XPS Measurements.** In the following section, it will be demonstrated that the stepwise adsorption of polyethylenimine and DOPA can also be followed by means of XPS measurements. Measurements have been performed on bare silicon wafers and on silicon wafers after adsorption of PEI and DOPA. The results are depicted in Figure 8. The O<sub>1s</sub> and the Si<sub>1s</sub> and the Si<sub>2p</sub> peaks are characteristic for the silicon wafers (Figure A). Although the cleaning procedure etches off part of the natural SiO<sub>2</sub> layer, the presence of a thin silicon oxide layer is obvious. This is also reflected by the fact that a small satellite peak of the Si<sub>2p</sub> was detected, which is characteristic for the Si-2p electrons in SiO<sub>2</sub>.<sup>42</sup> Also a slight contamination of the surface with carbon is detectable, which results from adsorption of contaminants such as CO<sub>2</sub> during transport to the apparatus. Figure 8B shows the XPS spectrum after the immersion of a cleaned silicon wafer in a solution of polyethylenimine. The intense peaks of silicon and oxygen are still detectable. In addition, strong signals from carbon and nitrogen are detectable, which reflect the presence of an adsorbed layer of polyethylenimine at the surface. As mentioned previously, the existence of this adsorption film of PEI could not be derived from X-ray



**Figure 8.** X-ray photoelectron spectra of (A) silicon wafer after purification, (B) silicon wafer being exposed to a solution of polyethylenimine for 30 min, and (C) silicon wafer after being exposed to a solution of polyethylenimine for 30 min and a solution of DOPA vesicles for 40 min.

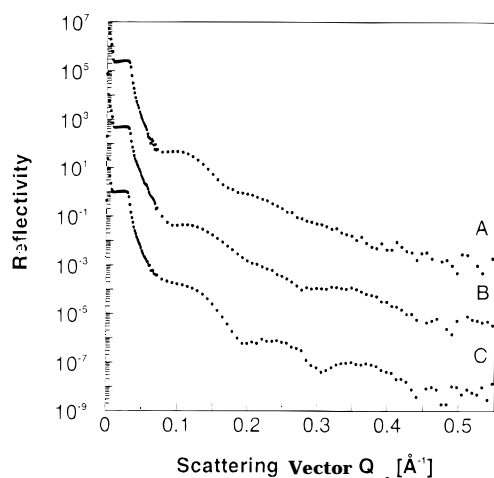
reflectivity measurements alone. The adsorption of a phospholipid layer (Figure 8C) on top of the polyelectrolyte layer can also be followed by means of XPS. After the last adsorption step the carbon peak is the most intense and the intensities of the silicon, nitrogen, and oxygen peaks have decreased. This proves again the layer structure of the adsorption film.

**2. Supported Bilayers.** It was reported above that a supported bilayer of DOPA can be created on polyethylenimine-treated surfaces by means of adsorption from liposome solutions. The second monolayer is floated off, when the sample is removed from the aqueous phase. The question arises whether the phospholipid bilayer can be stabilized by further adsorption of polyelectrolyte to create a structure as shown in Figure 1C. The scattering curves of three different samples with successive exposure to a polyethylenimine solution, a vesicle solution, and a further polyethylenimine solution are depicted in Figure 9.

The scattering curves of these systems differ from those with the supported monolayers (Figure 3). The first minimum is shifted to lower scattering angles, and the distance between successive minima has become smaller. The comparison of the three curves shows also that different samples differ more than in the case of the supported monolayers. Therefore no simulations were performed for the scattering curves. Assuming to a first approximation a layer with a homogeneous electron

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**Figure 9.** Measured reflectivity curves from three samples of supported bilayers after adsorption of polyethylenimine. The corresponding idealized structure is depicted in Figure 1C.

density, the total film thickness can be calculated from the position of the first minimum ( $Q_{\min}$ ) of the scattering curve as  $\pi/Q_{\min}$ . Thirty-eight angstroms was calculated for one sample (Figure 7, curve C), and 46 Å for two other samples (Figure 7, curves A and B). The approximate values for the film thicknesses are in all cases higher than those for the samples with supported monolayers. Assuming the same structure for the second phospholipid monolayer and the top polyethylenimine layer in the supported bilayers as in the supported monolayers, one would expect a total film thickness between 46.2 Å from the values in Table 1 (adsorption time of 5 min) and 51.8 Å (adsorption time of 40 min). Therefore the total thickness of 46 Å in the two cases suggests that a supported bilayer between polyethylenimine layers is created in the subphase, which remains on the substrate during drying and leads to an increased film thickness of the dried films. The lower value of 38 Å for the third sample can be explained by two different models: one possibility is that in this case a second bilayer phase has been formed in which the phospholipid layers are interdigitated. Such phases have already been detected for other polyelectrolyte/phospholipid complexes which are formed by precipitation from solution.<sup>21</sup> A second explanation is favored in which it is assumed that a supported bilayer with a non-interdigitated phase with polyelectrolyte on top is formed, with the bilayer structure being incomplete. Therefore, part of the sample surface is covered with a supported monolayer. This is supported by the observation that the samples do not exhibit a complete hydrophilic surface. This could also be caused by a partial floating off of a part of the second monolayer during the washing or drying process. The different number of defects leads to the differences in the diffractograms of the three samples. It has to be pointed out that by determining the layer thickness of the dried films it is not possible to derive direct information about the electron density profile. Therefore the increased layer thickness is only an indirect proof that films with an idealized structure, schematically depicted in Figure 1C, have been formed in the subphase. It cannot be excluded that a partial rearrangement of the supported bilayers takes place in such a way that parts of the alkyl chains are directed to the air/film interface in order to minimize the interfacial energy of the films.

In the following, the different behavior of the supported bilayers during washing and drying with and without the adsorption of an additional PEI layer will be discussed and arguments are given that at least parts of our films can maintain the structure shown in Figure 1C after

drying. Without an additional PEI layer, the top phospholipid monolayer can desorb easily and for example form a monolayer at the air/water interface, when the films are removed from the aqueous phase or during the washing steps. In such a way, supported monolayers of PEI on DOPA were obtained. The observed behavior of the supported bilayers is typical for monomeric phospholipids in general, as the corresponding supported bilayers after preparation are always handled in an aqueous phase for characterization, for example by means of surface force measurements<sup>43</sup> or surface plasmon spectroscopy.<sup>14</sup>

The higher stability of the supported bilayers with PEI adsorbed can be explained with the specific adsorption and desorption behavior of high molecular weight polymers and polyelectrolytes. The supported bilayers can be destroyed by two processes during washing. The first would be the successive desorption of PEI and DOPA, the second the desorption of the outer polyelectrolyte/lipid complexes to form a monolayer at the air/water interface. In both cases polymeric species are desorbed. It is typical for the adsorption behaviors of polymers in dilute solutions on surfaces that by diluting the solution the polymers cannot be removed from the surface in many cases. Due to the van der Waals interaction of a large number of segments with the surface, the theoretically calculated affinity for the surface is so high that extremely strong dilution is necessary to achieve desorption.<sup>44</sup> For polymers of about a hundred segments, already typical values of one molecule per cubic kilometer are obtained. As already mentioned before, a strong adhesion is also found for polyelectrolytes to oppositely charged surfaces.<sup>20–29,33,39</sup> In the system under investigation a stepwise desorption of PEI and DOPA during washing is improbable, as there are strong interactions between the charges of the ammonium groups of polyethylenimine and the phosphate groups of the lipids. The stability of these species has been demonstrated above, where it was found that a DOPA monolayer stays adsorbed on PEI on silicon even after three washing steps. Therefore the most probable desorption process is the desorption of the DOPA/PEI complex to form a monolayer at the air/water interface during washing, as the two DOPA monolayers are only held together by van der Waals interactions. It is expected that the van der Waals interaction per alkyl chain between the two DOPA layers does not change much by adsorption of PEI. But the DOPA/PEI complexes adhere with a large number of alkyl chains on the monolayer adjacent to the substrate and therefore exhibit a higher affinity for the monolayer surface. This makes the desorption of the outer monolayer much more difficult than the desorption of single DOPA molecules which are not bound to PEI (Figure 2B) and explains the observed SAXR results.

These arguments are supported by the results in two other publications in which the supported bilayers have been prepared by means of the Langmuir–Blodgett technique: Lowack and Helm have prepared supported bilayers by a combination of chemisorption and Langmuir–Blodgett transfer.<sup>45</sup> First, a supported monolayer has been formed by reaction of octadecyltrichlorosilane with the silicon surface. In the next step, a second monolayer consisting of a mixture of a biotin phospholipid and a poly(methacrylate) copolymer with hydrophobic and hydrophilic substituents was transferred by means of Langmuir–Blodgett deposition. By SAXR measurements it could be demonstrated that by this preparation method

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a supported bilayer had been obtained and that the latter stayed stable even after drying. Additionally, by means of specific protein binding it was shown that the hydrophilic headgroups of the biotin lipid dissolved in the polymeric matrix remained on the formerly water-adjacent side of the monolayer, even if this side had been exposed to air. This was explained by the fact that the formerly water-adjacent side of the top monolayer is hydrophobic also, and it was suggested that due to the limited flexibility of the polymer backbone some apolar groups are found already at the polymer/water interface before LB deposition. A system which is more similar to ours was investigated by Lvov et al.<sup>22</sup> In this study, Langmuir–Blodgett transfer was combined with polycation/polyanion self-assembly of polyelectrolytes.<sup>22</sup> Langmuir–Blodgett films could be obtained from monolayers at the air/water interface of the lipid/polyelectrolyte complex DODAB/PVS (dimethyldioctadecylammonium bromide/poly(vinyl sulfate)). The Langmuir–Blodgett films of DODAB/PVS with headgroups/polyelectrolyte on top on a precursor film on a solid substrate could be removed from the subphase through a monolayer free compartment of the film balance and washed for three times. Afterward further adsorption steps of oppositely charged polyelectrolyte and deposition of further LB films were been performed. The results of the SAXR measurements showed that by this method intact Langmuir–Blodgett bilayers with headgroups on top of the films could be successfully incorporated in the superstructures under investigation. In accordance with the experiments of Lowack and Helm<sup>45</sup> and especially of Lvov et al.,<sup>22</sup> the results of our investigations suggest a stronger adhesion of the top monolayer on the substrate-adjacent monolayer in supported bilayers of polymeric lipids and complexes of charged lipids with polyelectrolytes compared with monomeric lipids. This can be explained with the specific adsorption/desorption behavior of polymers.

## Conclusions

It has been demonstrated that supported bilayers of dioleoyl-L- $\alpha$ -phosphatidic acid can be formed by means of adsorption from solutions of vesicles onto polyethylenimine-treated substrates. On removal of the substrates from the aqueous phase, supported monolayers are left which could be characterized by means of SAXR and XPS measurements. By simulation of the reflectivity (SAXR) curves, the electron density profiles of the supported monolayers could be determined. The existence of the PEI monolayers at the silicon substrate could be derived from the ESCA measurements. By successive adsorption of polyethylenimine, DOPA vesicles, and again polyethylenimine, supported bilayers of lipid polyelectrolyte complexes could be created. Compared with top monolayers of the pure phospholipid bilayers on the PEI-treated substrates, the top lipid/polyelectrolyte monolayers show an increased adhesion to the substrate adjacent layers, which is reflected in an increased film thickness of the dried films, as detected by SAXS. Future experiments on this system will have to focus on the investigation of the fluidity of the supported bilayers, for example by means of FRAPP measurements and additional structural details such as the water content in the polyelectrolyte layers.

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